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Plasma urolithin metabolites correlate with improvements in endothelial function after red raspberry consumption: a double-blind randomized controlled trial

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ABSTRACT

Raspberries are a rich source of ellagitannins and anthocyanins. The aim of this work was to investigate whether raspberry consumption can improve vascular function and to understand which phenolic metabolites may be responsible for the effects. A 3 arm double-blind randomized controlled crossover human intervention trial was conducted in 10 healthy males. Flow-mediated dilation (FMD) was measured at baseline, 2 h, and 24 h post-consumption of 200 g and 400 g of red raspberries containing 201 or 403 mg of total (poly)phenols, or a matched control drink. Raspberry (poly)phenol metabolites were analyzed in plasma and urine by UPLC-QTOF mass spectrometry using authentic standards. Significant improvements in FMD were observed at 2 h (1.6% (95%CI 1.2, 1.9) and 1.2% (95% CI 0.8, 1.5)) and 24 h (1.0% (95% CI 0.6, 1.2) and 0.7% (95%CI 0.2, 0.9)) post-consumption of the 200 and 400 g raspberry drinks as compared to control, respectively. Plasma ellagic acid, urolithin A-3-glucuronide and urolithin A-sulfate correlated with the improvements in FMD at 2 and 24 h post consumption, respectively. Consumption of dietary achievable amounts of red raspberries acutely improves endothelial function up to 24 h and ellagitannins may be responsible for the observed effect.

KEYWORDS

Raspberries, endothelial function, (poly)phenols, ellagittanins, urolithins

ABBREVIATIONS

area under the curve, AUC; augmentation Index, AI; cardiovascular disease, CVD; coronary artery disease, CAD; coronary heart disease, CHD; flow-mediated dilation, FMD; pulse wave analysis, PWA; pulse wave velocity, PWV; randomized controlled trial, RCT; total (poly)phenols, TP.

INTRODUCTION

Red raspberries are one of the most common berry fruits consumed in the US with a yearly average availability of 560 g per capita in 2015 and still increasing in popularity today (https://www.ers.usda.gov). Despite being a very popular fruit, to our knowledge no clinical study has been published investigating the vascular effects of red raspberries in human subjects, although a number of preclinical studies have indicated potential health benefits (1-4).

Red raspberries are good sources of putatively bioactive (poly)phenolic compounds, including ellagitannins and anthocyanins (5, 6), which have been associated with lower cardiovascular disease (CVD) risk according to recent meta-analyses (7, 8). Evidence from randomized controlled trials with ellagitannin-rich sources (i.e. strawberries, pomegranate, and walnuts) have shown improvements in surrogate biomarkers of cardiovascular risk such as blood lipids, glycemic index, and blood pressure in at-risk individuals (9-14). The bioavailability of (poly)phenols in plasma is mainly characterized by an early absorption in the small intestine followed by a late colonic uptake. As early as 1 h after red raspberry consumption, ellagic acid was reported in plasma in low nanomolar concentrations (15). Most of the ellagitannins, however, travel further down the intestine and reach the colon after a few hours where they are transformed into urolithin catabolites by colonic microbiota (16). In contrast to other phenolic compounds, urolithins remain in the circulation for up to 80 hours after consumption (17, 18). Anthocyanins, also present in red raspberries, were shown to be absorbed and metabolized – both in the early and later phases - with main phenolic breakdown products and metabolites identified in urine and plasma as cinnamic acids, hippuric acids, phenylacetic acids, phenylpropionic acids, and benzoic acids (19, 20).

In the current study, we aimed to investigate the potential vascular benefits of red raspberries in healthy humans and identify which of the berry (poly)phenol metabolites may be responsible for the effects.

SUBJECTS AND METHODS

Study subjects

Ten healthy male volunteers aged 18 to 35 years were recruited from the University of Düsseldorf and surrounding area. Health was ascertained by a routine clinical physical exam and specific cardiovascular history performed by a cardiovascular specialist. Manifest cardiovascular disease including coronary artery disease, cerebrovascular disease, and peripheral artery disease, diabetes mellitus, acute inflammation, terminal renal failure, malignancies, and heart rhythm other than sinus were exclusion criteria.

Study Design

A three-arm, double blind, crossover randomized controlled trial was conducted. Informed consent was obtained and subjects were randomized to the treatments. We investigated the vascular effects of 200 g and 400 g of frozen red raspberries compared with a matched control drink. Measurements were taken at baseline, at 2 h, and at 24 h post-acute consumption. Blood samples were drawn at all time points for measuring plasma raspberry (poly)phenol metabolites. A 24 h urine sample was collected throughout each of the study days. Volunteers were instructed not to alter their usual dietary or fluid intake. The 10 volunteers selected for the study were asked to refrain from the following: consumption of (poly)phenol-rich foods including fruits, vegetables, cocoa, chocolate, coffee, tea and wine 24 h prior to the study, participating in vigorous exercise (> 3 x 20 min per week) and consuming more than 168 g of alcohol (any form) per week. Compliance to the diet and lifestyle restrictions was determined via a 24 h-dietary recall and via interview. At the end of the clinical exams, i.e. after completion of 2 h measurements, all subjects were given low nitrate mineral water and foods low in (poly)phenols

for lunch and dinner (lunch consisted of chicken slices, cheese, one boiled egg, and a low fat yoghurt; dinner consisted of ready made macaroni and cheese). They were instructed to drink water ad libitum, to eat only the given foods and to overnight fast for at least 12 hours prior to the 24 h measurement was made on the following day.

The primary endpoint was an improvement of endothelial vasodilator function as measured by flow-mediated vasodilation (FMD) using high-resolution ultrasound. Secondary endpoints were improvements in key determinants of vascular function and included decreases in pulse wave-velocity (PWV), aortic augmentation index (AIX), and blood pressure (ambulatory and central) as determined automatically by a blood pressure monitoring system and applanation tonometry (Sphygmocor). Tertiary endpoints include the quantification of plasma raspberry-derived (poly)phenols and were subsequently correlated with the primary and secondary end points (pre-specified analysis).

Office blood pressure was measured three times after 10 min of rest using an automated clinical digital sphygmomanometer (Dynamap, Tampa, FL, USA) with appropriately sized cuff placed around the upper arm at heart level.

A qualified researcher enrolled participants on the study. Participants and researchers administering interventions and assessing study outcomes were blinded to the interventions. An independent researcher generated the random allocation to treatment sequence (using a Williams design) and implemented the allocation sequence. Unblinding was performed after analysis of primary and secondary outcomes was completed. An independent researcher in possession of the blinding codes sent the codes via email to the researchers. Written informed consent was obtained from all subjects prior to their participation in the study. All studies were conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving

human subjects were approved by the University of Duesseldorf Research Ethics Committee (ref: 5327R). The study was also registered with the National Institutes of Health (NIH)-randomized trial records held on the NIH ClinicalTrials.gov website (NCT02734901). This study was conducted from March 2016 until September 2016. Volunteer recruitment started in March 2016 and was completed within a month. Study visits ran from March 2016 until May 2016 without changes in trial protocol/outcomes. Data collection was performed between April and September 2016 and statistical analysis were performed in September 2016.

Raspberry and control test drinks

Drinks were freshly prepared in the mornings of the study visits and were served in opaque bottles with black straws. Frozen *rubus ideaeus* (same batch for the whole study) were purchased at the supermarket (EDEKA, Duesseldorf) and stored at -20℃. The drink containing 400 g of raspberries was prepared by blending 400 g of frozen raspberries with 100 ml of water. The drink containing 200 g of raspberries was prepared by blending 200 g of frozen raspberries with 300 ml of water, soluble and insoluble fibers (Pectin, Natura, Brazil; cellulose, Nutricology, USA, respectively), vitamin C (Clasikool, UK), glucose (Thornton and Ross, UK), fructose (Special Ingredients, UK), and citric acid (Hexeal chemicals, UK), to match it to the 400 g raspberry drink (final volume of both drinks was 592 ml). The placebo drink was micro- and macronutrient matched to the 400 g raspberry drink, and had the same colour and taste. It was made by blending soluble and insoluble fibers, sugars, citric acid, red colorant (Red40, Heitmann, Germany), synthetic raspberry flavor (Lorann Oils, USA) and 550 ml of mineral water. The intervention drinks were administered on the study days between 7:00 and 11:00 am in the presence of the researcher to ensure compliance.

Raspberry (poly)phenol analysis

Freeze-dried raspberry powder was weighted and extracted with 1.5 mL of MeOH:H2O (50:50 v/v). The samples were then sonicated and centrifuged at 15000 g for 15 min at 4℃. A second extraction was performed on the pellet with 1 ml of the same extraction solvent (total volume=2.5 ml). After combining supernatants from both extraction steps, samples were filtered through 0.22 µM PVDF filters and injected directly in the UPLC.

Total ellagitannins were quantified after acid hydrolysis of raspberry samples using a method previously reported (21, 22). The method was based on the quantification of the acid hydrolysis products that include ellagic acid and valoneic acid dilactone. The hydrolysis products were quantified with the calibration curve of ellagic acid at 360 nm.

Ultrasound measurements of arterial function and pulse wave analysis

FMD was measured as previously described (23). Briefly, the diameter and flow velocity of the brachial artery (BA) was measured using a 12 MHz transducer (Vivid I, GE) and automatic edge-detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA, USA) yielding standard deviations of mean differences between repeated measurements of less than 1%. BA diameter was measured 2 cm proximal to the elbow. Reactive hyperemia was induced by 5 min of distal lower arm occlusion with a sphygmomanometric cuff inflated to 250 mm Hg. After cuff deflation at 0, 20, 40, 60, and 80 sec, the diameter was assessed and FMD calculated as maximal relative diameter gain relative to baseline. The FMD was expressed as $(diameter_{max}\text{-}diameter_{baseline})/diameter_{baseline} \times 100$.

Central blood pressure parameters including augmentation index (AIX) and pulse wave velocity (PWV) were measured by applanation tonometry using the SphygmoCor® (SMART medical,

Gloucestershire, UK) system. Via a transfer function, the pressure waveform of the ascending aorta was synthesized. PWV was determined from measurements taken at the carotid and femoral artery as previously described (24).

Biochemical analyses

The blood samples collected in EDTA/heparin tubes were spun (1,700 *g*; 15 min; 4ºC) immediately after collection. Plasma samples for (poly)phenol analysis were spiked with 2% formic acid and frozen at -80°C until analysis. All clinical chemistry parameters including total, LDL-, and HDL-cholesterol, triglycerides (enzymatic photometric assay; RocheDiagnostics), Hb_{A1c}, glucose (hexokinase assay) and whole blood count (flow cytometry; Sysmex) were measured using standard techniques by the Institute for Clinical Chemistry, University Hospital Duesseldorf, Germany.

UPLC-Q-TOF MS analysis of plasma and urine (poly)phenols

Plasma and urinary analysis of polyphenol metabolites was performed using microelution solid phase extraction coupled with UPLC-Q-TOF MS and authentic standards for quantification as previously described, using a validated method (25). For the identification and quantification of urolithins, the microbial metabolites of ellagitannins, previously validated methods with UPLC-QTOF MS for plasma samples and HPLC-DAD-Q MS for urine samples were used (26). A target screening strategy was applied to all the samples for the qualitative screening of possible ellagic acid derived metabolites. Quantification of urolithins was determined by interpolation in the calibration curve obtained with their own available standards by peak area integration of its extracted ion chromatograms. Calibration curves of a mixture of all urolithins (urolithin A 3-

glucuronide, isourolithin A 3-glucuronide, Urolithin A sulfate, urolithin B glucuronide , urolithin B sulfate, Urolithin D, urolithin M6, urolithin M7, isourolithin A, urolithin A, and urolithin B) were prepared in methanol. Total (poly)phenol (TP) concentrations were calculated based on the sum of all phenolic compounds (59 in plasma and 67 in urine) quantified excl. urolithins and ellagic acid.

Power calculation and statistical analysis

Power calculations were performed for the primary endpoint, change in FMD response, using an online statistical tool (http://hedwig.mgh.harvard.edu/sample_size/size.html). Power was based on the standard deviation of the difference between two values for the same patient (intra-individual variability) of the operator that performed the FMD analysis (SD=1%). Based on previous work, we would expect to see a change in FMD of 1-2% (19, 27, 28). At 0.8 power and at 0.05 significance level, the number of subjects required to detect a difference of 0.720% in the response of matched pairs in a crossover study is 10. The characteristics of the study population are expressed as mean values and standard deviations. Changes in FMD values and changes in plasma and urinary (poly)phenol concentrations were analyzed by one-way ANOVA with Bonferroni correction. Correlation analysis was performed with Pearson or Spearman tests depending on normal and non-normal distribution, respectively. Mean values of results are presented as mean values and their standard error of means, and differences between responses are presented as mean values and 95% confidence intervals. Analyses were computed with Prism 7 and SPSS 20 (IBM).

RESULTS

Baseline characteristics of the study population and tolerance of intervention

The baseline characteristics of the groups of healthy young non-obese males were all within normal limits **(Table 1).** All study subjects completed the study, drinks were well tolerated by all subjects, and no adverse events were reported.

(Poly)phenol content of the red raspberry drinks

A total of 27 (poly)phenolic compounds were quantified in the raspberry drinks used in the present study, including 2 ellagitanins, 2 anthocyanins, 5 flavonols, 2 flavan-3-ols, 8 cinnamic acids, 6 benzoic acids, and 2 benzaldehydes **(Table 2)**. In 200 g and 400 g of raspberries, 201 mg and 403 mg of (poly)phenolic compounds were quantified, respectively. As expected, ellagitannins and anthocyanins were the most abundant compounds found in the red raspberries.

Red raspberry acutely improves endothelial function and this is maintained at 24 h after consumption

FMD increased significantly by 1.6% (95% CI 1.2%, 1.9%) and 1.2% (95% CI 0.8%, 1.5%) at 2 h post-consumption of the raspberry drink containing 200 g and 400 g of raspberry, respectively, when compared with the change in FMD due to control drink (Figure 2). When FMD was measured at 24 h after consumption of 200 g or 400 g raspberries, following a strictly (poly)phenol-free controlled diet and an overnight fast (12 h), significant increases of 1.0% (95% CI 0.6%, 1.2%) and 0.7% (95% CI 0.2%, 0.9%) were found as compared to changes due to control (Figure 2). The FMD changes due to 200 g and 400 g raspberry did not significantly

differ. No significant changes in peripheral and central blood pressure, PWV, and AIX were observed between or within treatments at 2 h or 24 h post-consumption **(Table 3)**.

Identification and quantification of ellagitannin metabolites in plasma and urine

A total of 15 ellagitannin metabolites were identified in plasma and urine, including ellagic acid and derivatives, urolithins A, B, C and their glucuronide and sulfate conjugates as well as isourolithin A with corresponding glucuronide and sulfate conjugates. Of these, seven metabolites were quantified using authentic standards (Supplementary Table 1). Results from plasma analysis showed that ellagic acid increased significantly at 2 h post-consumption of the raspberry drinks, in comparison with control or baseline (Figure 3). At 24 h, urolithin-A-3-glucuronide, urolithin B-glucuronide, urolithin-A-sulfate, urolithin-B-sulfate and total urolithins significantly increased after consumption of 200 g and 400 g raspberries as compared to baseline and control drink (Figure 3). Isourolithin-A-3-glucuronide also increased significantly ($p<0.0001$) in plasma at 24 h after consumption of the raspberry drinks but was only found in 2 volunteers (4.2 nM and 15.9 nM after 200 g and 400 g raspberries, respectively). Plasma urolithin C was only found in one volunteer at 24 h post-consumption of 400 g of raspberries at a very low concentration (1.0 nM). Other plasma metabolites were identified in some volunteers but not quantified due to the lack of authentic standards: urolithin-A sulfoglucuronide (n=9, 24 h), urolithin-C sulfate (n=4, 24 h), dimethyl ellagic acid glucuronide (n=9, 2 and 24 h), and dimethyl ellagic acid (n=1, 2 and 24 h).

Urinary analysis confirmed the presence of urolithin-A, urolithin-A-3-glucuronide, urolithin-A-sulfate, isourolithin-A, isourolithin-A-glucuronide and urolithin-B-glucuronide. Total urolithins were excreted in significantly higher amounts when comparing 400 g raspberry (5.4 ± 8.4 mg)

versus control intake (0.1 ± 0.1 mg, p=0.02) over a period of 24 h (Supplementary table 1). Table 5 shows a summary of 24 h urinary excretions of urolithin metabolites. Significant increases in urolithin-A and urolithin-B-glucuronide were observed after intake of 400 g of raspberries as compared to control. Iso-urolithin-A-3-glucuronide showed significant increases for both the 200 and 400 g intakes with respect to control. Urolithins had a urinary recovery of 7% and 9% after intake of 30 mg and 60 mg of ellagitannins respectively (table 5). Out of the ten volunteers, eight were identified as metabotype A, with urolithin-A-3-glucuronide and urolithin-A sulfate as the main metabolites and some traces of ellagic acid, whereas two of them were identified as metabotype B with the presence of isourolithin-A-3-glucuronide, urolithin-B glucuronide and urolithin-B sulfate in addition to urolithin-A derivatives.

Identification and quantification of raspberry derived phenolic metabolites in plasma and urine

Using authentic standards, a total of 59 phenolic metabolites were quantified in plasma (Supplementary Table 1). Most of the metabolites were conjugated and non-conjugated phenolic acid derivatives. The total quantified plasma (poly)phenol (TP) concentration was 105±7 μM (mean±SEM) and 109±10 μM at 2 h and 24 h after consumption of 200 g of raspberries. After intake of 400 g of raspberries, the TP levels were 112±10 μM at 2h and 120±12 μM at 24 h. TP were significantly increased (p=0.04) at 24 h after consumption of 400 g of raspberries when compared to control. Two metabolites (4-methylgallic-3-*O*-sulfate, and 3,4-dihydroxybenzaldehyde) were significantly increased at 2 h after 400 g of raspberry intake. Dihydroferulic acid 4-*O*-β-D-glucuronide, ellagic acid, and pyrogallol-1-*O*-sulfate were significantly elevated as compared to control after 2 h of 200 g raspberry intake. Protocatechuic

acid-4-*O*-sulfate and ellagic acid showed significant increases 24 h after 400 g of raspberry in comparison to control. A total of 67 phenolic metabolites were detected in the urine of the volunteers after intake of raspberries (Supplementary Table 1).

Ellagitannin-derived phenolic metabolites correlate with the increase in endothelial function

To link the circulating metabolites with vascular effects, we performed a correlation analysis between changes in plasma (poly)phenol concentrations and changes in FMD. At 2 h post-consumption of both raspberry drinks, ellagic acid correlated with FMD. At 24 h post-consumption, urolithin-A-3-glucuronide and urolithin-A-sulfate correlated with FMD, but only after consumption of the 200 g raspberry drink (Table 4 and Supplementary Figure 1).

Discussion

In summary, the current study demonstrate for the first time that the consumption of red raspberries can increase endothelial function for 24 h and that this effect is associated with patterns of circulating ellagitanin metabolites in healthy humans.

In the search for the mechanisms of action of dietary (poly)phenols and causality assumptions related to this, it is essential to consider the pharmacokinetics of these compounds. Most studies investigating the acute effects of (poly)phenols on endothelial function have reported an early improvement at 1 to 2 h post-consumption coinciding with peak plasma concentrations of the phenolic compounds that are, upon ingestion, rapidly absorbed in the small intestine and reach circulation intact or conjugated by phase II enzymes (24, 29). However, we have recently shown that (poly)phenol consumption can lead to improvements in FMD for up to 8 hours post-consumption (27, 29). At this time, the majority of the circulating (poly)phenol metabolites are likely gut microbiome derived phenolic acid metabolites. After blueberry consumption, FMD increased biphasically with a first peak at 1-2 h that correlated with intestinally absorbed and metabolized phenolic compounds and a second peak at 6 h that correlated with gut microbial metabolites (19), and the effects were maintained up to 8 hours post consumption of cranberry juice (29). It must be noted that no significant differences were found between FMD improvements after consumption of the 200 and 400 g of raspberries. This agrees with our previous work, where the effect on FMD plateaued after the consumption of the equivalent to 240 g of blueberries (19). A similar non-linear dose response on FMD was found after consumption of cranberry juice (29), and in a meta-analysis of randomized controlled trials investigating the effects of flavonoids on FMD (25)**.**

The effects that we are reporting here at 2 and 24 hours after consumption coincide with the C_{max} of ellagic acid and urolithins, respectivedly (See Figure 3 and (17)**).** This indicates that the vascular effects of raspberries may be driven both by early readily absorbed metabolites and late gut microbial metabolites of raspberry polyphenols. Ellagic acid was detected in plasma at 2 h post-consumption, in line with previously reported plasma ellagic acid detection (low nM range) after 1 h of ellagitannin-rich pomegranate consumption (15). Furthermore, we show that at 24 h post consumption of red raspberries, urolithin A-sulfate and urolithin A-3-glucuronide reached higher concentrations in both plasma and urine with respect to baseline and 2h time point (Figure 3). Interestingly, amongst all phenolic compounds quantified upon red raspberry consumption, only the early and late phase absorbed ellagitannin-derived metabolites correlated with improved endothelial function in healthy humans, despite many other compounds, in particular the ones derived from anthocyanins, present in much higher concentration in plasma. To our knowledge, this is the first time that plasma urolithin A metabolites are linked to improvements in vascular function as they are present in the blood vessels at the same time as the vascular measurements and the magnitude of the effects correlates with changes in vascular function. In agreement with our findings, recently published *in vitro* work has shown that a mix of urolithin metabolites significantly increased nitric oxide (NO) bioavailability (30), and urolithin-A and urolithin-A-glucuronide significantly decreased monocyte adhesion and inhibited endothelial cell migration *in vitro* (31). This suggests that in particular the late vascular effects of red raspberry may be caused by gut microbiome derived phenolic metabolites and, therefore, the gut microbiome may play an important role in mediating beneficial effects of raspberries in healthy humans. It should be noted that the concentration of urolithin metabolites used in these in vitro studies, although physiologically relevant, were higher than the ones shown here in the plasma of volunteers.

Significant increases in urinary excretions of urolithins were observed for both the 200 and 400 g of raspberry consumption over control (Table 5). Higher concentrations of urolithins were excreted after consumption of the 400 g raspberries in comparison with the 200 g. This is in agreement with our previous work showing that circulating levels of (poly)phenol metabolites did not reach a plateau despite an obvious saturation of vascular effects (19, 29).

Metabotypes for urolithin production have been recently determined as type A (urolithin A production), B (isourolithin A and B production) and 0 (non-producers) (32), and differences in gut dysbiosis and cardiovascular disease risk have been shown among different metabotypes (33). Obese or overweight subjects, and individuals with metabolic syndrome or colorectal cancer had a higher percentage of B metabotype than healthy individuals (34). A recent study reported that only overweight or obese individuals with metabotype B showed improvements in blood lipids after consumption of ellagitannin-rich pomegranate extract for 3 weeks (33). However, individuals with metabotype B had higher baseline levels of total and LDL cholesterol than individuals with metabotype A, which could be the reason why significant changes were observed only in that group. In our healthy cohort of young normoweight males, we did not see any differences in baseline characteristics, and although very speculative, we observed a non-significant trend towards increased FMD response in metabotype A (n=8) volunteers as compared to metabotype B volunteers (n=2). Whether individuals with different metabotypes may have a different vascular response to ellagitannins deserves further investigation.

Notable limitations of this work are that the study population consisted of a small group of healthy young men. Therefore, our findings cannot be directly extrapolated to all segments of the general population. Furthermore, the study was limited in scope and timeframe and, therefore,

further studies with larger cohorts and over a longer timeframe are needed to investigate whether the acute effects of raspberry ellagitannins on cardiovascular disease risk biomarkers translate into health benefits. Administration of purified extracts or purified (poly)phenols underlines the identification of bioactive metabolites, which is why we remain cautious with the interpretation that bioactive urolithin metabolites may improve vascular health, as pure ellagitannins were not included in the study design as a third parallel arm.

In conclusion, or present data show for the first time that the consumption of dietary achievable amounts of red raspberries by healthy individuals lead to a clinically relevant improvement in endothelial function for at least 24 h after consumption, which according to recent meta-analysis would imply a reduction of 10-15% in CVD risk (35, 36) if sustained over a long enough timeframe. Furthermore, we linked gut microbial derived plasma urolithin metabolites to vascular benefits. Further studies will need to show whether our results translate into long-term health benefits in the general population and whether this is also true for other ellagitannins-rich foods besides red raspberries - strawberries, pomegranate, or nuts – that may be included as part of a healthy diet to aid in the prevention of cardiovascular disease.

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Conflict of Interest:

There were no conflicts of interest.

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TABLES

Table 1: Baseline clinical characteristics study population (n=10).

	Mean ± SD
Age (years)	27 ± 3
Weight (kg)	78 ± 8
BMI (kg/m²)	23 ± 2
SBP (mmHg)	124 ± 10
DBP (mmHg)	68 ± 6
HR (/min)	58 ± 7
TRIG (mg/dl)	110.3 ± 35.2
HDL-C (mg/dl)	53 ± 9.3
LDL-C (mg/dl)	104 ± 19.1
CRP (mg/dl)	0.2 ± 0.1
GLUC (mg/dl)	86.4 ± 7.9

Table 2: (Poly)phenolic, macro and micronutrient composition of the intervention drinks

Raspberry amount (g)	**0 (Control)**	**200**	**400**
Total (poly)phenols (mg)	0	201	403
Total ellagitannins (mg)	0	30	60
Ellagic acid derivatives (mg)	0	23	45
Valoneic acid dilactone (mg)	0	7.8	15.5
Total anthocyanins (mg)	0	164	328
Cyanidin-3-sophoroside (mg)	0	125	251
Cyanidin-3-glucoside (mg)	0	39	77
Total flavonols (mg)	0	5.7	11
Quercetin (mg)	0	0.03	0.05
Quercetin-sophoroside (mg)	0	2.3	4.6
Quercetin pentoside (mg)	0	2.2	4.4
Quercetin-3-glucuronide (mg)	0	1.2	2.4
Total cinnamic acids (mg)	0	0.7	1.4
p-Coumaric acid (mg)	0	0.02	0.04
o-Coumaric acid (mg)	0	0.03	0.06
Caffeic acid (mg)	0	0.01	0.02
Ferulic acid (mg)	0	0.02	0.04
Isoferulic acid (mg)	0	0.58	1.1
Sinapic acid (mg)	0	0.03	0.06
Chlorogenic acid (mg)	0	0.00	0.01
Total flavan-3-ols (mg)	0	0.6	1.2
Catechin (mg)	0	0.01	0.02
Epicatechin (mg)	0	0.60	1.2
Total benzoic acids (mg)	0	0.06	0.11
2-Hydroxybenzoic acid (mg)	0	0.01	0.02
3-Hydroxybenzoic acid (mg)	0	0.01	0.01
2,5-Dihydroxybenzoic acid (mg)	0	0.00	0.01
Protocatechuic acid (mg)	0	0.01	0.02
Gallic acid (mg)	0	0.02	0.04
Total benzaldehydes (mg)	0	0.02	0.03
3,4-Dihydroxybenzaldehyde (mg)	0	0.01	0.03
Mineral water (ml)	550	350	150
Glucose (g)	7.4	7.4	7.4
Fructose (g)	9.4	9.4	9.4
Fiber (g)	17.4	17.4	17.4
Citrate (g)	8	8	8
Vitamin C (g)	0.105	0.105	0.105
Total volume (ml)	592	592	592

Table 3: Effects of intervention raspberry drinks on vascular function.

	Control			200 g raspberries			400 g raspberries			Difference (Δ200 g - Δcontrol)		Difference (Δ400 g - Δcontrol)	
	Baseline	2h	24h	Baseline	2h	24h	Baseline	2h	24h	2h	24h	2h	24h
FMD (%)	6.6±0.4	6.6±0.4	6.7±0.5	6.4±0.4	8±0.5	7.4±0.5	6.6±0.5	7.8±0.4	7.2±0.5	1.6 (1.2, 1.9)*	0.9 (0.5, 1.3)*	1.2 (0.8, 1.5)*	0.5 (0.2, 0.9)*
PSBP (mmHg)	127.2±9.9	126.1±11.4	122.4±9.9	124.6±9.8	121.2±10.0	123.0±9.4	120.7±12.8	121.4±9.1	118.5±13.7	2.3 (-4.1, 8.7)	-3.1 (-9.5, 3.2)	4.0 (-2.4, 10.4)	-0.6 (-7.0, 5.8)
PDBP (mmHg)	70.8±7.8	68.8±8.3	64.7±8.0	69.6±7.6	68.9±6	68.3±9.5	68.7±6.5	69.7±8.2	66.8±9.0	-1.3 (-6.7, 4.1)	-4.9 (-10.3, 0.5)	1.7 (-3.7, 7.1)	-0.7 (-6.1, 4.7)
CSBP (mmHg)	105.5±8.3	102.7±9.1	102.2±9.6	105.1±8.4	101.2±7.4	101.9±5.7	102.7±10.0	102.5±7.6	99.3±9.6	0.2 (-4.7, 5.1)	-1.0 (-5.9, 3.9)	2.8 (-2.1, 7.7)	-1.1 (-6.0, 3.8)
CDBP (mmHg)	71.4±8.0	69±8.2	66.9±10.2	70.9±7.1	70.2±6.3	69.8±7.4	69.4±7.1	73.5±10.7	68.7±9.0	-2.4 (-7.8, 3.0)	-4.1 (-9.5, 1.3)	4.1 (-1.3, 9.5)	-0.3 (-5.7, 5.1)
HR (bpm)	60.9±8.2	56.8±6.1	60±7.6	58.5±10.0	55.4±7.2	61.7±11.1	56.9±7.9	56.8±7.4	60.3±8.4	-1.1 (-8.1, 5.9)	-4.2 (-11.2, 2.8)	3.0 (-4.0, 10.0)	0.2 (-6.8, 7.2)
PWV (m/s)	5.4±0.9	5.3±0.7	5.6±1.0	5.0±0.9	5.1±0.7	5.6±0.8	5.5±0.8	5.2±1.0	5.4±1.1	0.8 (-0.8, 2.5)	0.8 (-0.9, 2.5)	0.4 (-2.1, 1.3)	0.6 (-2.3, 1.1)
AIx (%)	-5.8±17.0	-12.7±9.8	-9.3±8.7	-9.2±6.3	-11.7±10.7	-8.1±8.2	-6.2±11.1	-12.0±10.2	-10.7±7.1	-6.3 (-17.5, 4.9)	-6.5 (-17.7, 4.7)	-5.2 (-16.4, 6.0)	-7.5 (-18.7, 3.7)

Outcomes are expressed as absolute values with SD and as difference of the changes with respect to baseline with 95% CI. Testing was done with one-way ANOVA calculations (Bonferroni correction) comparing changes with respect to baseline between treatments for each time point. * represent values that do not span zero and are thus significant. A significant difference in FMD was observed for the 200 g and 400 g raspberry intake as compared to control. No significant differences in FMD were observed between the 200 and 400 g raspberry intakes. FMD, flow-mediated dilation; PSBP, peripheral diastolic blood pressure; PDBP, peripheral systolic blood pressure; CSBP, central systolic blood pressure; CDBP, central diastolic blood pressure; HR, heart rate; PWV, pulse-wave velocity; AIx, augmentation index.

Table 4: Plasma (poly)phenols and vascular function correlation analysis.

	(Poly)phenols	Δ FMD 2 h		Δ FMD 24 h	
		ρ	**p**	**ρ**	**p**
200 g raspberry (n = 10)	Ellagic acid	0.78	0.01	-	-
	Urolithin-A-3-glucuronide	-	-	0.64	0.05
	Urolithin-A-sulfate	-	-	0.80	0.01
400 g raspberry (n = 10)	Ellagic acid	0.51	0.02	-	-

Significant correlations ($p<0.05$) between plasma raspberry derived (poly)phenol metabolites and changes in FMD with respect to baseline at 2 h or 24 h after consumption of 200 and 400 g raspberry drinks by healthy volunteers. Correlations are presented as Spearman's rho (**ρ)** for non-parametric data.

Table 5: Urinary excretion of urolithin metabolites.

	Control drink (0 mg ellagitannins) µg	200 g raspberries (30 mg ellagitannins) µg	400 g raspberries (60 mg ellagitannins) µg
Urolithin-A (n = 10)	0 ± 0	14 ± 0	172 ± 88**
Urolithin-A sulfate (n = 10)	0 ± 0	50 ± 0	295 ± 0
Urolithin-A-3-glucuronide (n = 10)	525 ± 232	1,488 ± 223	4,282 ± 2,757
Urolithin-B-glucuronide (n = 2)	0 ± 0	1,894 ± 57	2,985 ± 2,145**
Iso-urolithin-A (n = 2)	0 ± 0	6 ± 0	27 ± 0
Iso-urolithin-A-3-glucuronide (n = 2)	0 ± 0	965 ± 614*	1,716 ± 567***
Total urolithins (n = 10)	525 ± 232	2,131 ± 465	5,420 ± 2,812
Recovery (%)		7 ± 2	9 ± 5

Excretions were calculated based on the amount of total ellagitannins taken by the volunteers (30 and 60 mg). Urolithin-A and Urolithin-B-glucuronide showed significant increases after 400 g of raspberries with respect to control. Iso-urolithin-A-3-glucuronide showed significant increases in both doses with respect to control. One-way ANOVA with Bonferroni correction was used to determine significance. Marked results with asterixes indicate significant differences in excreted amounts over control. No significant differences in excretion were observed between the two doses *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$. Values are mean ± SEM.

Figure captions

Figure 1: Consort study flow (A) and study design (B) schemes

Figure 2: Changes in flow-mediated dilation (FMD) respect to baseline after consumption of the raspberry drinks containing 0, 201, and 403 mg of (poly)phenols. Significance was tested using repeated measurements two-way ANOVA with Bonferroni corrections where **p<0.01 and ****p<0.0001 significantly different from control. Values are represented as Mean ± SEM (n = 10).

Figure 3: Timecourse of the main ellagitannin metabolites quantified in plasma after intake of control, 201 mg and 403 mg total (poly)phenols(TP): A) total urolithins, B) ellagic acid, C) urolithin A sulfate, D) urolithin A-3-*O*-glucuronide, E) urolithin B glucuronide, F) urolithin B sulfate. Significance was tested using a repeated measures two-way ANOVA with Bonferroni corrections where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as significantly different from control. Values are represented as Mean ± SEM (n = 10).

Supplementary Material captions:

Supplementary Table 1: (Poly)phenol plasma concentrations (nM) and urinary excretions after intake of 400 g of raspberries. Values are represented as Mean ± SEM (n = 10)

Supplementary Figure 1: Individual correlation plots of ellagitannin metabolites with changes in FMD (Table 4): A) Ellagic acid (2 h after intake of 200 g raspberries), B) Ellagic acid (2 h after intake of 400 g raspberries), C) Urolithin A-sulfate (24 h after intake of 200 g raspberries and D) Urolithin A-glucuronide (24 h after intake of 200 g raspberries. Values are represented in nM concentrations (n = 10). Correlation analysis of the non-parametric data was assessed using a Spearman's test.